NEW COMPOSITION

FIELD OF THE INVENTION

This invention relates to the field of treatment and risk assessment for atherosclerosis and ischemic cardiovascular diseases.

DESCRIPTION OF RELATED ART

Atherosclerosis is a chronic disease that causes a thickening of the innermost layer (the intima) of large and medium-sized arteries. It decreases blood flow and may cause ischemia and tissue destruction in organs supplied by the affected vessel. Atherosclerosis is the major cause of cardiovascular disease including myocardial infarction, stroke and peripheral artery disease. It is the major cause of death in the western world and is predicted to become the leading cause of death in the entire world within two decades.

The disease is initiated by accumulation of lipoproteins, primarily low-density lipoprotein (LDL), in the extracellular matrix of the vessel. These LDL particles aggregate and undergo oxidative modification. Oxidized LDL is pro-inflammatory, toxic and causes vascular injury. Atherosclerosis represents in many respects a response to this injury including inflammation and fibrosis.

In 1989 Palinski and coworkers identified circulating autoantibodies against oxidized LDL in humans. This observation suggested that atherosclerosis may be an autoimmune disease caused by immune reactions against oxidized lipoproteins. At this time several laboratories began searching for associations between antibody titers against oxidized LDL and cardiovascular disease. However, the picture that emerged from these studies was far from clear. Antibodies existed against a large number of different epitopes in oxidized LDL, but the structure of these epitopes was unknown. The term "oxidized LDL antibodies" thus referred to an unknown mixture of different antibodies rather than to one specific antibody.

It is well established that there is an ongoing inflammation in the atherosclerotic lesions, characterized by activation of immune competent cells and production of

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inflammatory cytokines. Established risk factors like hypertension, blood lipids, diabetes and smoking are likely to promote this inflammatory reaction, but the mechanism by which this occurs are not well characterized and different non-mutually exclusive possibilities exist. Several different autoantigens that could
5   elicit this immune reactivity have been proposed, including oxidized low density lipoprotein (oxLDL) and heat shock proteins (HSP)[2,3]. Available data on the role of immune reactions in atherosclerosis indicate a complex relationship. One example of this is immunization in animal models to influence atherogenesis. When HSP is used, atherosclerosis increases but decreases when oxLDL is the
10   antigen[4,5].

The role of aOxLDL in human disease appears to be complex. In humans, it has previously been demonstrated that aOxLDL is higher in healthy controls than in men with borderline hypertension, an example of early cardiovascular disease[6].
15   Recent studies are in line with this observation[7,8]. On the other hand, several authors have reported that aOxLDL are raised in human cardiovascular diseases (CVD), especially at later stages[2,3,9,10.] One example is systemic lupus erythematosus (SLE) and autoimmune disease associated with a very high risk of CVD. SLE-patients with a history of CVD had clearly raised aOxLDL –levels[11].
20   These to some extent contradictory results may depend on different methods and stages of LDL-oxidation, yielding differences in antigenicity. It is also likely that disease stage and risk factor profile are related to antibody levels.

Oxidized low density lipoprotein (oxLDL) itself has many proinflammatory
25   properties including activation of T cells[12,13], monocytes/macrophages and endothelial cells[14-16]. OxLDL promotes inflammation also in immune competent cells from atherosclerotic lesions[17]. However, it should be noted that oxLDL may also ameliorate acute inflammatory reactions and instead promote a more low-grade chronic inflammation as that seen in atherosclerosis[18]. It is interesting to
30   note that many biological effects of oxLDL are caused by platelet activating factor (PAF)-like lipids in oxLDL[19-21]

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Phosphorylcholine (PC) is a major component not only in inflammatory phospholipids like platelet activating factor-PAF (where it is essential for interaction with the PAF-receptor) and in oxLDL, but is also as an immunogenic components of many bacteria including *S. Pneumoniae*[22]. Furthermore, PC is expressed by apoptotic cells[2,23].

In US5455032 phosphocholine conjugates have been used in vaccines for inducing immunoprotection against infections such as *Streptococcus pneumoniae*. In a recent study [24] by Binder et al on pneumococcus vaccine in mice, it was also shown that vaccination decreased atherosclerotic lesion formation. It was found that many autoantibodies to oxLDL derived from atherosclerotic mice share structural identity with antibodies which protect against common infectious pathogens, including *Streptococcus pneumoniae* . The study, in mice, not humans, does not give any information about specificity, or that IgM anti-phosphorylcholine antibodies are significantly more important than corresponding IgG antibodies as a protecting factor in atherosclerosis. Furthermore, phosphorylcholine conjugates have not been used in the pneumococcus vaccine.

In another study it was shown [25] that antiphosphorylcholine antibody levels are elevated in humans with periodontal diseases. The conclusion is that phosphorylcholine is an important oral antigen associated with organisms in the periodontal flora and that anti-PC antibody is elevated as a consequence of periodontal disease. No information is given with regards to the antibodies and possible protection from or progression of atherosclerosis.

A couple of documents (e.g. WO2002080954 and WO0168119) related to immunization treatment of atherosclerosis have been published but these are either based on the use of peptide fragments of apolipoprotein B or antibodies to alpha/beta chains of a T cell receptor. A method to detect atherosclerotic plaque (WO9908109) using monoclonal antibodies to oxidation-specific epitopes on lipoprotein has also been described. This is different from the method proposed in this invention where a phosphorylcholine conjugate is used to detect antibodies, eg IgM or IgG antibodies, in subject samples.

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SUMMARY OF THE INVENTION

5   The invention relates to pharmaceutical compositions comprising a phosphorylcholine conjugate, or an antibody preparation, for example a monoclonal antibody, with specificity to a phosphorylcholine conjugate, and the use of these compositions in the treatment or prevention of atherosclerosis, for example in the treatment, prevention or reduction of further progression of

10   atherosclerosis Furthermore, the invention also relates to the use of phosphorylcholine conjugates or said antibody preparation, for example monoclonal antibody to produce a pharmaceutical composition optionally with an adjuvant. Furthermore the invention relates to diagnosing the presence or absence of antibodies, for example IgM or IgG antibodies, related to increased or

15   decreased risk of developing ischemic cardiovascular diseases.

A first aspect of the invention provides the use of a pharmaceutical composition comprising at least one phosphorylcholine conjugate, or an antibody preparation, for example a monoclonal antibody, with specificity to a phosphorylcholine

20   conjugate, in the manufacture of a medicament for immunization and treatment of mammals, including humans, against atherosclerosis or an atherosclerotic related disease.   The medicament is intended to provide immunization having immunogenic or therapeutic properties against atherosclerosis.

25   A second aspect of the invention provides a method for immunization and treatment of a mammal, including a human, against atherosclerosis or an atherosclerotic related disease, the method comprising the step of administering to the mammal a pharmaceutical composition comprising at least one phosphorylcholine conjugate, or an antibody preparation, for example a

30   monoclonal antibody, with specificity to a phosphorylcholine conjugate.   The pharmaceutical composition is intended to provide immunization having immunogenic or therapeutic properties against atherosclerosis.

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By phosphorylcholine conjugate is meant a phosphorylcholine moiety linked to a carrier, preferably via a spacer. The structural element phosphorylcholine may comprise a derivative of phosphorylcholine. Examples of suitable phosphorylcholine conjugates are described in US 5,455,032, as noted

5   above. For example, US 5,455,032 provides phosphorylcholine conjugates in which the phosphorylcholine moiety is linked by a straight chain alkyl and an amide linkage to a variety of immunological carriers. The phosphorylcholine conjugate may for example be a human serum albumin (HSA)- or keyhole limpet hemocyanin (KLH)-phosphorylcholine conjugate or a bovine serum albumin

10   (BSA)-phosphorylcholine conjugate (for example as described in the Examples). PC-BSA (Phosphorylcholine-Bovine Serum Albumin) can be purchased from Biosearch Technologies, INC (Ca, USA). HSA-BSA-can be conjugated by a chemical procedure, for example the following procedure:

15   O-(4-aminophenyl phosphoryl)-choline (I) can be prepared from O-(4-nitrophenyl-phosphoryl) -choline (Sigma N 5879) in quantitative yield by reduction with hydrogen gas at 1 atm with 10 % palladium at charcoal as a catalyst, according to a procedure described by Chesebro, B. in Biochemistry 11,(1972) 766 .

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(I) can be coupled to HSA by means of EDC (1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide) in MES buffer pH 4 ,essentially according to a procedure described by Padilla, N.D.et al in J.Immun..Methods 293 (2004) 1-11. The conjugated HSA can isolated by dialysis against buffered saline at pH 7.4.

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The carrier can be, for example, a protein, lipid or polymer. The carrier can be latex beads, for example as described in the Examples.

The medicament may be intended for administration by injection.

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A further aspect of the invention provides the use of one or more of the phosphorylcholine conjugates as defined in relation to the preceding aspects of the invention, in the manufacture of a pharmaceutical composition, optionally in

combination with an adjuvant, for immunotherapy or therapy for the treatment of ischemic cardiovascular diseases.

A further aspect of the invention provides a method of prophylactic or therapeutic treatment of a mammal, which may be a human being, suffering from atherosclerosis or facing the risk of developing ischemic cardiovascular disease, whereby a therapeutically effective amount of at least one phosphorylcholine conjugate or an antibody preparation, for example a monoclonal antibody, with specificity to a phosphorylcholine conjugate is administered.

The invention also relates to methods to determine the presence or absence of antibodies, for example IgM or IgG antibodies, against phosphorylcholine which are related to an increased or decreased risk of developing ischemic cardiovascular diseases.

A further aspect of the invention provides a method of diagnosing the presence or absence of antibodies, for example IgM or IgG antibodies, related to increased or decreased risk of developing ischemic cardiovascular diseases, using a phosphorylcholine conjugate.

Thus, a further aspect of the invention provides the use of a phosphorylcholine conjugate in a method for assessing a patient's risk of developing or progression of ischemic cardiovascular disease in which the patient's levels of antibodies, for example IgM or IgG antibodies, reactive with the phosphorylcholine conjugate are assessed.

Phosphorylcholine conjugates are described above. The phosphorylcholine may be linked to a carrier via a spacer. The carrier may be a protein, which may be KLH (keyhole limpet hemocyanin) or human serum albumin (HSA). The carrier may be latex beads.

The patient's levels of antibodies, eg IgM or IgG antibodies, reactive with the phosphorylcholine conjugate may be assessed using an immunoassay. Examples

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of suitable immunoassays are described below and will in any case be apparent to those skilled in the art.

It may be desirable to measure antibodies reactive with oxLDL or MD-LDL as well as measuring antibodies, eg IgM or IgG antibodies, reactive with the phosphorylcholine conjugate. It may alternatively or in addition be desirable to measure levels of HSP70, HDL, TNF and/or HSP60 (as discussed in the Examples) as well as measuring antibodies, eg IgM or IgG antibodies, reactive with the phosphorylcholine conjugate.

DETAILED DESCRIPTION OF THE INVENTION

The examples disclosed below are provided only for the purpose of illustrating the present invention and should not be considered as any limitation of the scope as outlined in the appended claims. Document referred to herein are hereby incorporated by reference.

An example of a method to determine the presence or absence (or level) of IgM antibodies against phosphorylcholine which is related to an increased or decreased risk of developing ischemic cardiovascular diseases is described. Other methods known in the art can also be used. Similar methods may be used to determine the presence or absence (or level) of IgG antibodies against phosphorylcholine.

Methods to determine the presence or absence of IgM antibodies against phosphorylcholine

IgM antibodies to PC-BSA were determined by an enzyme-linked immunsorbent assay method.

A microtiter plate was coated with PC-BSA (10μg/ml; for example from Biosearch Technologies, INC (Ca, USA) in phosphate buffered saline (PBS). After washings with PBS, the plates were blocked with a 2% BSA solution. Serum samples were diluted (1:30) in 0.2% BSA-PBS . Plates were incubated overnight

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at 40°C and washed. Alkaline phosphatase conjugated goat anti-human IgM (diluted 1:7000 in the sample buffer) were added at 100ul/well and incubated at 40°C overnight. After washings, colour was developed by adding an alkaline phosphatase substrate and incubating the plates for 60 min at room temperature in the dark. The absorbances were read in a spectrophotometer at 405nm.

Different carriers and spacers for phosphorylcholine have been tested. The exemplified carriers are not limited to these. Other carriers such as other proteins, lipids or polymers, such as latex beads which are known in the art, may also be used. Carriers are discussed in US 5,455,032, as noted above.

The IgM or IgG antibodies detected by a method of the invention may also bind to phosphorylcholine (PC) present in PC-containing compounds in which PC is exposed, for example in lysophosphatidylcholine (lysoPC; see, for example, Kim et al, J Exp Med. 2002 Sep 2;196(5):655-65). Thus, a method of the invention may detect IgM or IgG antibodies that bind to lysphosphatidylcholine.

Synthesis of a phosphorylcholine conjugate and preparation of a pharmaceutical composition

Latex beads (0.20 μm or 0.81μm) were suspended in PBS and mixed over night with a 10 μg/ml solution of phosphorylcholine-BSA. The beads were then centrifuged and washed several times with buffer and blocked with a 10μg/ml solution of BSA. After another repeated washing, the beads were resuspended to a suitable concentration in a suitable buffer and stored refrigerated until use. Phosphorylcholine with a linker arm can also be conjugated to KLH (keyhole limpet hemocyanin) via a diazophenyl group. More preferably a p-nitrophenyl-6-(O-phosphocholine)hydroxyhexanoate derivative of PC can be synthesized according to Chesebro, B. and Metzger, H. (1972) Biochem. 11:776. p-Nitrophenyl-6-(O-phosphocholine) hydroxyhexanoate was dissolved in dry acetonitrile (100 mg/ml) just prior to adding it to the KLH. Derivative and KLH were mixed overnight at 4° C and then dialyzed to remove unbound spacer and p-nitrophenylate, which is the leaving group.

An injection solution of the prepared phosphorylcholine conjugate, suspended in a suitable buffer, can be directly used for immunization.

5   Immunization with a phosphorylcholine conjugate

A high titer of IgM antibodies recognizing phosphorylcholine was determined in plasma from BALB/c mice after immunization with 200 µg [p-Nitrophenyl-6-(O-phosphocholine) hydroxyhexanoate –KLH] i.p. using the suggested immunoassay method.

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Monoclonal antibodies against a phosphorylcholine conjugate

Monoclonal antibodies can be produced using any standard method known in the art. See for example "Briles DE, Forman C, Hudak S, Claflin JL. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against

15   Streptococcus pneumoniae. *J Exp Med*. 1982;156:1177-85" or "T15 PC binding monoclonal antibodies retain specificity when they switch from IgM to IgG. , Spira, Gad; Aguila, Hector L.; Scharff, Matthew D.   Fac. Med., Techniton-Israel Inst. Technol., Haifa, Israel. *Journal of Immunology* (1988), 140(8), 2675-80.

20   Other antibodies against a phosphorylcholine conjugate can be prepared using methods well known to those skilled in the art. For example, a subfraction with aPC activity of a human immunoglobulin preparation can be prepared, for example as described below, for example by affinity purification using a phosphorylcholine conjugate.   Intravenous immunoglobulin preparations (e.g.

25   IGIV; Baxter and others) is a highly purified preparation of IgG commercially available and is used in the treatment of patients who have no, or very low levels of antibody production.   Immunoglobulin preparations include those available from the following manufacturers: Baxter (US) eg Gammagard®, Isiven (Antimo Naples, Italy), Omrix (Tel-Hashomer, Israel), Miles (Biological Products Division,

30   West Heaven, CT), Sclavo (Lucca, Italy), Sandoz (Novartis, Basel, Swizerland) eg Sandoglobulin®, Biotest Diagnostic Corporation (Deville, NJ).   Examples of immunoglobulin preparations are Gammagard S/D®, Gammar IV®, Gammar-P IV®,   Gammimune   N®,   Iveegam®,   Panglobulin®,   Polygam   S/D®,

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Sandoglobulin®,   Venoglobulin®. Immunoglobulin preparations typically contain some IgM as well as IgG.   Trace amounts of IgM are present in Gammagard®. Pentaglobin (Biotest) is an enriched IgM preparation which has been used for treatment of SARS.   The subfraction with aPC activity may

5   comprise both IgG and IgM, or may be selected to comprise mainly IgG (for example by starting with an IgG-rich preparation such as Gammagard® and/or by selecting for IgG); or mainly IgM (for example by starting with an IgM-rich preparation such as Pentaglobin and/or by selecting for IgM).

10   An antibody preparation with specificity to a phosphorylcholine conjugate binds to unconjugated phosphorylcholine and may also bind to phosphorylcholine (PC) present in PC-containing compounds in which PC is exposed, for example in lysophosphatidylcholine (lysoPC; see, for example, Kim et al, J Exp Med. 2002 Sep 2;196(5):655-65).   Thus, an antibody preparation with specificity to a

15   phosphorylcholine conjugate may also bind to lysphosphatidylcholine.

IgM immunoglobulin levels in atherosclerotic subjects

IgM autoantibody levels against phosphorylcholine in subjects with hypertension (diastolic pressure>95 mmHg) were determined at baseline and after 4 years in a

20   correlation study of risk factors for atherosclerosis. The results are summarized below.

Carotid plaques were detected in 77 subjects (35%) at enrolment, and in 84 subjects (38%) at the 4-year follow-up. In total 218 human subjects were in the study. Increases in intima-media thickness (IMT) at follow-up were less prevalent

25   in subjects having high serum levels of IgM to PC (75th or 90th percentile) at the time of enrolment. There is a significant difference between mean values in IgM anti-phosphorylcholine antibody levels between individuals with increased and decreased IMT (638.8±219.6 vs. 734.8±266.9, p=.004).

The relationships between IgM autoantibodies to PC and changes in IMT were

30   independent of age, smoking habits, treatment with atenolol or lacidipine and blood lipids. IgM autoantibodies were also independent of IgG values.

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One embodiment of the present invention is thus to use a phosphorylcholine conjugate for the preparation of a pharmaceutical composition to be used in the treatment or prevention of atherosclerosis. The conjugate can be phosphorylcholine linked to a protein or to a polymer. The pharmaceutical composition is preferably given by injection.

The proposed method of active immunization will modulate the autoantibodies titer which in turn will have a positive effect on the development of atherosclerosis.

Another embodiment of the invention is to use an antibody preparation, for example a monoclonal antibody, recognizing a phosphorylcholine conjugate for the preparation of a pharmaceutical composition to be used in the treatment or prevention of atherosclerosis. The monoclonal antibody can be produced using methods known in the art.

A further embodiment of the invention is to provide a method of diagnosing the presence or absence of antibodies, for example IgM or IgG antibodies, towards phosphorylcholine which factor is related to an increased or decreased risk of developing ischemic cardiovascular diseases, using a phosphorylcholine conjugate. A preferred method is an immunoassay. The method may be used in assessing the patient's risk of developing or progression of ischemic cardiovascular disease.

Figures:

Figure 1a: Inhibition of antibody (IgM) bdining to ELISA-plates coated with PC albumin by β2GPI, PS and CL. Inhibition by different antigens of binding to PC albumin-coated plates. In order to investigate the specificity of aPC, competition assays were performed as described in the Experimental section. Results are presented as mean ±SD.

Figure 1b: Inhibition of antibody (IgG) binding to ELISA-pates coated with PC albumin by β2GPI, PS and CL. Inhibition by different antigens of binding to PC albumin-coated plates. In order to investigate the specificity of aPC, competition assays were performed as described in the Experimental section. Results are presented as mean ±SD.

Figure 2a: Inhibition of antibody (IgM) binding to ELISA-pates coated with PC albumin by oxLDL and MDA-LDL. Inhibition by different antigens of binding to

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PC albumin-coated plates. In order to investigate the specificity of aPC, competition assays were performed as described in the Experimental section. Results are presented as mean ±SD.

Figure 2b: Inhibition of antibody (IgG) binding to ELISA-pates coated with PC albumin by oxLDL and MDA-LDL. Inhibition by different antigens of binding to PC albumin-coated plates. In order to investigate the specificity of aPC, competition assays were performed as described in the Experimental section. Results are presented as mean ±SD.

Figure 3: effect on oxLDL uptake in macrophages by aPC extracted from IGIV

We tested two groups: macrophages with oxLDL and macrophages with oxLDL + preincubation with aPC extracted from IVIG.

macrophage+ -oxLDL (total 107 cells): Weak staining 37/107 = 34.58%Strong Staining   10/   107=   9.35%

total   staining   positive   47/107=   43.93%

macrophage+Dil-oxLDL+   aPC-   group   (checked   156   cells):

Weak   staining   37/156   =   23.72%

Strong   Staining   2/   156=   1.28%

total staining positive 39/156= 25%.

Figure 3A shows staining with Dil-labelled oxLDL. Figure 3B shows staining with unlabeled oxLDL.

Figure 4: Effect of pre-incubation of high antiphospholipid antibodies (aPLs) titer serum with human pooled immunoglobulin Gammagard® on Annexin V binding to human umbilical endothelia cells (HUVECs): flow cytometry analysis after 24 hrs culture.

IVIG pre-incubated with serum at	Median fluorescence intensity (MFI) of Annexin V binding
0 mg/ml	649

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2.5 mg/ml	913
5 mg/ml	1269
10 mg/ml	1382

Figure 5: effect of aPC on ICAM-induction in endothelial cells

1 ug/ml of PAF was added to cultures of endothelial cells, with or without preincubation with aPC IgM. Expression of ICAM-1 was tested by FACScan. Green line represents PAF effect, while red is PAF + aPC IgM and black control. The data clearly indicate a shift to the left of histogram when aPC IgM are added.

EXPERIMENTAL

Subjects

Serum samples were obtained from 226 subjects with established hypertension (diastolic pressure >95 mm Hg) prior to their entry into the Swedish component of the European Lacidipine Study on Atherosclerosis (ELSA)[25,26]. Samples were collected following a 4-week washout period with no medication to minimize the effects of treatment on the measured parameters. Blood pressure, cholesterol and triglyceride levels were determined as described previously[25,26]. One hundred and fifteen of the subjects were subsequently assigned to treatment with the β-blocker atenolol, and 111 of the subjects were assigned to treatment with the calcium antagonist lacidipine. The study was approved by the Ethics Committee of the Karolinska Hospital and was conducted in accordance with the Helsinki Declaration. All subjects gave informed consent.

Carotid ultrasound

Carotid ultrasound determinations were performed and analysed as detailed elsewhere25,26. A total of 218 patients had valid ultrasound measurements at baseline and 4-year follow up. Briefly, the right and left carotid arteries were examined with Biosound 2000 IIA duplex scanner using an 8.0 MHz annular array transducer. The intima-media (I-M) thickness was determined in the far wall as the distance between the leading edge of the lumen-intima echo and the leading

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edge of the media-adventitious echo. The outcome measurement as a surrogate indicator for atherosclerosis was the change in mean maximum Intimal-Medial thickness (IMT) of the four far walls in the distal common carotids and carotid bifurcations bilaterally (CBMmax) at the 4-year follow-up. The associations between antibody levels to PC at enrolment into the study with an increase or decrease in IMT at the 4-year follow-up were evaluated.

Reagents

Polysorp F96 microtiter immuno-plates were purchased from Nunc (Roskilde Denmark), PC-BSA (Phosphorylcholine-Bovine Serum Albumin) was purchased from Biosearch Technologies, INC (USA).

Bovine serum albumin (BSA), Alkaline phosphatase conjugated goat anti-human IgG(r-chain specific) , Alkaline phosphatase conjugated goat anti-human IgM(u-chain specific), PNPP(Alkaline phosphatase substrate), were obtained from Sigma (St. Louis, MO, USA). Cardiolipin (CL) was purchased from AVANTT (US, $\beta_2$glycoprotein (β_2GP1) was obtained from Calbiochem (US).

Total IgG and IgM levels were determined by routine techniques as previously described[6].

CRP was analyzed in erum by highly sensitive methods using particle-enhanced immunonephelometry (Behring Nephelometer Analyzer, BN II (Dade Behring GmBH, Marburg, Germany)) with an antir-assay variation <4%.

Determination of autoantibodies against PC, oxLDL and MDA-LDL

IgG and igM antibodies to PC-BSA were determined by enzyme-linked immunsorbent assay (ELISA). Pooled serum from 17 antiphospholipid syndrome patients was used as an internal standard and tested on every plate. The plateau of antibody binding was reached with the antigen concentration of 10µg/ml. F96 microtiter polysorp pate was therefore coated with PC-BSA (10µg/ml) 50 µg.well in PBS. Coated plates were incubated overnight at 4°C. After five washings with PBS, the plates were blocked with 2% BSA-PBS for 2h at room temperature and washed as described above. Serum saples were diluted (1:30) in 0.2% BSA-PBS and added at 50µl/well.

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LDL was isolated from plasma of healthy donors by sequential preparative ultra-centrifugation and oxidized by use of copper ions (OxLDL) or derivatized with MDA(MDA-LDL) as described[6].

OxLDL and MDA-LDL were determined by ELISA essentially as described[6].

OxLDL or MDA-LDL was diluted to 2µg/ml in coating buffer (carbonate-bicarboatne buffer 50 mM pH9.7), and 100µl/well was used to coat ELISA plates (Costar 2581). The plates were kept at 4°C overnight, washed 4 times with PBS, and then blocked with 20% adult bovine serum in PBS (20% ABS-PBS) for 2 horus in room temperature. They were then incubated with 100µl serum, diluted 1:30 in 20% ABS-PBS at 4°C overnight.

Plates were incubated overnight at 4°C and washed as described above. Alkaline phosphatase conjugated goat anti-human IgG (diluted 1:9000 in the sample buffer) and Alkaline phosphatase conjugated goat anti-human IgM (diluted 1:7000 in the sample buffer) were added at 100µl/well and incubated at 4°C overnight. After five washing, color was developed by adding the alkaline phosphatase substrate (PNPP) at 100µl/well and incubating the plates for 60 min at room temperature int eh dark. The plates were read in an ELISA Multiskan Plus spectrophotometer at 405nm. All samples were measured in a single assay and the coefficient of variation was below 10-15%.

Specificity of anti-phosphorylcholine-BSA antibodies

In order to investigate the specificity of anti-phosphorylcholine-BSA, absorption assays were performed by use of pooled high titer sera. At a dilution giving 50% of maximal binding to PC-BSA, high titer pooled sera were preincubated with different concentration of PC-BSA. After vortexing, the tubes were incubated at 40°C overnight and centrifuged at 13000r.p.m. for 30 min(40°C) The supernmatants were tested for antibody binding to PC-BSA as described. The percentage of inhibition was calculated as follows:

Percent inhibition = (OD without competitor – OD with competitor) x100/ OD without competitor.

Statistical analysis

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anti-phosphorylcholine   levels   were   dichotomized   at   the   75th   and   90th

percentile. The association between anti-phosphorylcholine (or other antibodies)

and the progression of atherosclerosis over a 4-year period were determined by

estimating increases in IMT (yes or no) using logistic regression analysis and the

5   calculation of odds ratios (ORs) and 95% confidence intervals (CI), or comparison

using Spearman correlation as indicated. Adjustments were made for possible

confounders including age, smoking habits, serum cholesterol, serum triglycerides

and mode of anti-hypertensive treatment (lacidipine, atenolol). A two-tailed p-

value < 0.05 was considered as significant.

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Results

Basic characteristics of the subjects at the time of enrolment into the study have

been detailed elswhere (Pockley et al (2003) Hypertension 42, 235-238) and are

presented in Table I.

15   Competition studies reveal that aPC of IgM and IgG subclass was competed out

by   preincubation   with   PC-BSA,   while   cardiolipin   had   a   weak   and

phosphatidylserine no competitive capacity (Figure 1a, 1b). β2-glycoprotein I

competed to some extent with IgG binding to PC-BSA but not so much with IgM

(figure 1a, 1b). PC-BSA had a low capacity to compete out binding to the other

20   antigens tested (data not shown). OxLDL and MDA-LDL could compete out

binding of IgM aPC to PC-BSA, and also of IGG aPC, though not to the same

extent (Figure 2a, 2b).

Increases in IMT at follw-up were less prevalent in subjects having high serum

levels of IgM to PC (75th or 90th percentile), oxLDL and MDA-LDL (90th

25   percentile) at the time of enrolment, while CRP was not associated with IMT-

changes (Table 2).

Logistic regression analysis revealed that the relationships between IgM

autoantibodies to PC, oxLDL and MDA-LDL and changes in IMT were

independent of age, smoking habits, treatment with atenolol or lacidipine and

30   blood lipids. aPC IgM were significantly associated with changes in IMT at both

75th and 90th percentile, while aOxLDL and aMDA-LDL of IgM subclass only

showed significance at the 90th (table 3a-d). IgM autoantibodies were also

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independent of IgG values (data not shown). Furthermore, total IgG and IgM levels were not associated with IMT-measurements or changes (data not shown).

IgG autoantibodies to PC were trendwise lower in subjects with increases in IMT
5   but this difference did not reach statistical significance (Table 2).

There were striking differences ebtween men and women. aPC, aMDA-LDL, and aOxLDL of IgM subcalss, were significantly higher in women than in men (p's<0.05). In contrast, there were no differences between men and women in IgG levels of these autoantibodies. In addition, women had a significantly lower
10   occurrence of plaque at baseline and follow-up (p<0.05).

aPC IgM levels correlated negatively with increase in IMT (Rho 0.18, p=0.006) in contrast to two other protection factors, HDL and HSP70 which did not correlate with IMT changes as continuous measurements (data not shown). Unlike aPC
15   IgM, aOXLDL and aMDA-LDL did not reach significance in these determinations (data not shown).

There were significant associations between aPC IgM levels and aOCLDL IgM (Rho 0.74, p<0.001) and aMDA-LDL IgM (rho 0.51, p<0.001). Likewise aPC correlated with HSP60(Rho 0.28, p<0.001), HSP70 (Rho 0.35, p<0.001), which
20   we recently described as a novel protective afactor for human athersclerosis in this cohort (Pockley et al (2003) *supra*) and also with HDL (Rho 0.23, p<0.01). There were not associations between aPC IgM, aOxLDL IgM or aOxLDL, MDA-LDL and LDL, CRP or triglycerides (data not shown).

When separate logistic regression analyses were made for men and women,
25   controlling for age, total cholesterol, triglycerides, smoking and treatment IgM aPC showed significant rpotective effects in women only when 90th percentile was studied (EXP (B)=.17, 95% CI=0.05-.68; p=0.01 and in men only when 75th percentile was studied EXP (B)=.18, 95% CI=0.04-.74; p=0.01, respectively).

IgM to MDA-LDL and oxLDL differend in this respect, since only values for
30   women reached statistical significance independently. Thus, when separate logistic regression analyses were made for men and women, controlling for age,

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total cholesterol, triglycerides, smoking and treatment both IgM to MDA-LDL and IgM to OxLDL showed significant protective effects in women (EXP (B)=.17, 95% CI=.05-.68, p=0.01 and EXP (B) =.18, 95% CI = .04-.74, p=0.01, respectively), whereas the effect did not reach significance among men (EXP (B)=.60, 95% CI=.15-2.2, p=0.44, and EXP (B)=.39, 95% CI=.10-1.5, p=0.17 respectively), indicating that high IgM titers to OxLDL and to MDA-LDL may be specifically protective among women.

Table 1. Basic characteristics of the study group at enrolment. Results are presented as means (SD) or percentage (%) and mg/dL for lipids.

	Total (N=226)	Atenolol (N=115)	Lacidipine (N=111)
Age (years)	57.7 (7.8)	57.6 (7.6)	57.7 (7.9)
Sex (% males)	50	46	53
BMI	26.7 (3.7)	26.3 (3.3)	27.1 (3.9)
Total cholesterol	232.4 (37.8)	233.5(38.1)	231.4(37.4)
HDL	55.6 (27.6)	56.5(25.8)	54.7 (27.6)
LDL	149.4 (37.8)	149.7(37.1)	149.2 (38.6)
Trigyclerides	131.6 (58.2)	128.6 (57.0)	134.7 (59.5)

Table 2. Unadjusted prediction of changes in IMT with baseline levels of IgG and IgM autoantibodies to phosphorylcholine (PC).

Variable	Odds Ratio 75th percentile	(95% CI) Lower	Upper	P
aPC (IgG)	.60	.32	1.1	.10
aPC (IgM)	.46	.25	.85	.01
aOxLDL (IgG)	1.2	.64	2.3	.57
aOxLDL	.77	.41	1.4	.40

(IgM)				
aMDA-LDL (IgG)	.80	.43	1.5	.48
aMDA-LDL (IgM)	.67	.36	1.2	.18
C-reactive protein	.80	.43	1.5	.46
	90th percentile			
aPC (IgG)	.60	.25	1.4	.24
aPC (IgM)	.36	.15	0.87	.024
aOxLDL (IgG)	.94	.38	2.31	.90
aOxLDL (IgM)	.27	.11	.69	.006
aMDA-LDL (IgG)	.63	.26	1.5	.30
aMDA-LDL (IgM)	.27	.11	.69	.006
C-reactive protein	.60	.24	1.4	.24

Table 3a: prediction of changes in MT over a 4-year period using the 75th percentile of aPC IgM autoantibodies at baseline in subjects with established hypertension.

Variable in the model	Coefficient (B)	Estimated odds ratio Exp(B)	P	95% CI Lower	Upper
Smoking	-.01	.99	.95	.66	1.5
Sex	-.05	.95	.87	.54	1.4
Total cholesterol	.003	1.0	.45	.99	1.0

Plasma triglycerides	-.001	.99	.63	.99	1.0
Age (years)	.01	1.0	.59	.97	1.0
Treatment (A/L)	-.23	.79	.40	.45	1.4
APC IgM	-1.0	.37	.0027	.15	.89

Table 3b: prediction of changes in IMT over a 4-hyear period using the 90th precentile of aPC IgM autoantibodies in subjects with established hypertension.

Variable in the model	Coefficient (B)	Estimated odds ratio Exp(B)	P	95% CI Lower	Upper
Smoking	-.02	.97	.90	.65	1.5
Sex	-.005	1.0	.98	.56	1.8
Total cholesterol	.003	1.0	.42	.99	1.0
Plasma triglycerides	-.001	0.99	.67	.99	1.0
Age (years)	.003	1.0	.87	.97	1.0
Treatment (A/L)	-.22	.80	.43	.46	1.4
aPC IgM	-.77	.46	.017	.24	.87

Table 3c: Prediction of changes in IMT over a 4-year period using the 90th percentile with IgM autoantibodies to OxLDL and other risk factors in subjects with established hypertension

Variable in the model	Coefficient (B)	Estimated odds ratio Exp(B)	P	95% CI Lower	Upper
Smoking	-.01	.99	.95	.66	1.5
Sex	.001	1.1	.98	.56	1.8
Total cholesterol	.001	1.0	.72	.99	1.1

Plasma triglycerides	-.001	1.0	.71	.99	1.0
Age (years)	.01	1.0	.60	.97	1.1
Treatment (A/L)	-.28	.77	.35	.44	1.3
aOxLDL IgM	-1.3	.26	.008	.11	.72

Table 3d: Predicition of changes in IMT over a 4-year period using the 90th percentile with IgM autoantibodies to MDA-LDL and other risk factors in subjects with established hypertension

Variable in the model	Coefficient (B)	Estimated odds ratio Exp(B)	P	95% CI Lower	Upper
Smoking	-.07	0.93	.73	.62	1.4
Sex	-.001	.99	.99	.56	1.7
Total cholesterol	0.001	1.0	.78	.99	1.0
Plasma triglycerides	-.001	.99	.74	.99	1.1
Age (years)	0.01	1.0	.54	.97	1.0
Treatment (A/L)	-.27	.76	.34	.44	1.3
aMDM-LDL IgM	-1.1	.31	.01	.12	.79

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Study showing protective effect of aPC

In an observational study from Malmö (the Malmö Diet and Cancer Study), about 6000 out of 30000 subjects from the cohort were recruited for extensive cardiovascular investigations, including non-invasive assessment of subclinical atherosclerosis through ultrasound measurements of the carotids. In addition, additional cardiovascular risk factors were measured at baseline. These subjects have been followed for over 10 years with regard to the occurrence of new events of cardiovascular diseases (myocardial infarction, chronic coronary heart disease, atherotrombotic stroke). In order to assess the relative risks (calculated as relative hazards) with 95% confidence intervals, nested-case-control analyses (3 controls per case) were undertaken for low levels of antibodies against phosphorylcholine

(aPC-IgM). There were in total 145 CVD cases (mainly myocardial infarction (MI) and ischemic stroke) and 400 age and sex-matched controls. The cutoff level for aPC was 307 for the tenth percentile of aPC levels. There were in total 20 CVD cases with aPC levels below the tenth percentile (14%), and 34 controls (9%), corresponding to a relative hazard of 1,9 (95% CI 1,1-4,3). The corresponding number of male CVD cases below the tenth percentile of aPC was 16 (19%), and 25 control patients below this level (11%), corresponding to a relative hazard of 1,9 (95% CI 1,1-3,5). The number of female cases was too low to yield robust information on relative risks (see Tables 1 and 2). The results suggest that low aPC levels are predictive for the occurrence of cardiovascular disease in healthy subjects, and could act as markers for cardiovascular diseases.

Table 1. Descriptive statistics for aPC ($<10^{th}$ percentile)

		SEX				All	
		Males		Females			
		Case	Control	Case	Control	Case	Control
Below 10th pct							
No	n	68	206	57	160	125	366
	%	81	89	93	95	86	92
Yes	N	16	25	4	9	20	34
	%	19	11	7	5	14	9

Table 2. Univariate analysis of the influence of aPC ($<10^{th}$ percentile) on CVD by conditional logistic regression for all patients, males and females, respectively.

	Variable	p-value	Hazard ratio	95% Hazard Ratio Confidence Limits	
All patients	aPC	0.0308	1.939	1.063	3.536
Males	aPC	0.0262	2.181	1.097	4.338
Females	aPC	0.6556	1.331	0.379	4.676

Effects of aPC

Introduction

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By use of columns which are preabsorbed with PC-BSA, PC-KLH or Pneumococcal vaccine (Statens Serum Institute, Denmark), we extract antibodies with reactivity against these compounds. Levels of aPC IgG are raised in at least the two first of these. Small amounts of IgM may be extracted out of IVIG and then also run through the columns preabsorbed with the abovementioned antigens. Through this method we can obtain polyclonal human aPC of IgG and IgM subclass. Protein measurement indicates that aPC IgM levels of 0.5 mg / ml could be extracted. Using these antibodies we can test their functional properties using in vitro models:

1. Can preincubation of increasing concentrations of aPC IgM with oxidized LDL decrease binding and uptake in monocyte/macrophage cell line, THP1? Test systems with confocal microscopy and/or FACS can be used.

2. Can preincubation of increasing concentrations of aPC IgM with normal IgM as control, with PAF, lysophosphatidylcholine (LPC) inhibit induction of adhesion molecules ICAM on endothelial cells by these lipids? Also other cytokines can be tested using a commercial kit (several different cytokines; BioSource). Tests can make use of FACScan.

Cell culture

Cryopreserved pooled HUVECs at passage 2 were purchased from Cascade Biologics, Inc. (Portland, OR, USA). Cultures were maintained in EGM™ phenol red-free medium (Clonetics, San Diego, CA, USA), containing 2% of fetal bovine serum and supplements. The cells were incubated in 75 cm^2 flasks (TPP, AG, Trasadingen, Switzerland) at 37°C under humidified 5% CO_2 conditions.

All experiments were performed at passage 3 to 4. Cells were seeded at $2x10^4$ cells / ml density in 12-well plates (NUNC, Inc, Naperville, IL, USA) for flow cytometry analysis. After allowing 12-24 hours for attachment the cells were made quiescent in SFM for at least 12 hrs prior to treatment.

Monocytic cell line THP-1 was from AT&T (USA). Cells were maintained in RPMI with 10% FCS.

Preparation of aPC

Total IgM or IgG fraction was separated from commercially available pooled human immunoglobulin (Gammagard®) at 50mg/ml using HiTrap IgM or IgG columns(Amersham Biosciences). Antibodies against phosphorylcholine (PC)
5   were eluted after loading IgM or IgG fraction on NHS-Sepharose columns coupled to PC conjugated either to keyhole limpet protein (KLH)(1 or 5 mg/ml) or to bovine serum albumin (BSA) (1 mg/ml), followed by BSA-only column. PC-BSA (Phosphorylcholine-Bovine Serum Albumin) and PC-KLH was purchased from Biosearch Technologies, INC (Ca, USA). Eluted fractions were buffer-
10   exchanged on PD-10 columns and concentrated with Millipore Centricone® devices. Procedures were performed according to instructions given by manufacturers. The concentration of IgM aPC prepared was typically 50 µg/ml, and the concentration of IgG aPC was typically 30µg/ml.

15   *Scavenger binding and uptake of oxLDL by THP-1 derived macrophages*

Oxidized LDL (oxLDL) is prepared as described by incubation with copper ions. First, oxLDL is labeled with DiI (DiI-(1,1'- dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, Inc)) and diluted in Saline-EDTA buffer to 1mg/ml. After that, 2ml lipoprotein-deficient serum is
20   added for 1mg of oxLDL and then filtered (0.45um). 50ul DiI (3mg/ml) in DMSO is added for 1mg oxLDL and the mixture is incubated 15h, 37^{0}C and then dialyzed against several changes of saline-EDTA for 6h. After this the mixture is 0.45um filtered again.

Uptake of the oxLDL is studied with fluorescence/confocal microscopy. THP-1
25   cells as models for monocytes/macrophages are grown overnight on slide chamber.(medium: DMEM/10% FBS/Glu/PEST)

3X wash with DMEM medium without FBS

Incubated with oxLDL-DiO 5ug/ml (SFM medium) 6h.

The cells washed with 0.2% BSA-PBS 5X, PBS 1X
30   Macrophage nucleus staining: the cells were incubated with 1ug/ml bisbenzimide 10' and wash with PBS 3X.

Fix and mount: the cells were then fixed with 4% paraformaldehyde in PBS for 30', PBS 3X, finally, after 1 drop of mounting gel. The slides were covered with cover slip.

Annexin V binding to endothelial cells

Heparin-preserved plasma with high capacity to inhibit Annexin V binding was added to HUVECs monolayer at concentration of 10% in SFM. After 24 hrs cells were harvested with Cell Dissociation Solution (CDS; Sigma-Aldrich, St. Louis, MO, USA) and carefully pooled with supernatants, to exclude selective loss of detached floating cells, centrifugation at 1200 rpm for 7 min followed. After resuspension in 100µl of annexin V-binding buffer (Molecular Probes Inc, Eugene, OR, USA) samples were stained with 2µl 5 mg/ml annexin V-FITC (Molecular Probes) and incubated for 15 min on ice. Shortly before acquisition 1 mg/ml of propidium iodide (PI; a vital dye; R&DSystems Europe Ltd, Abingdon, UK) was added. Analysis was performed as described above.

Statistical analysis

The statistics were computed using Stat View software, SAS Institute AB, Göteborg, Sweden. Skewed continuous variables were logarithmically transforme. Study groups were compared using ANOVA for continuous variables and Chi square for categorical variables. Fischer's PLSD was used as post hoc test. Correlation coefficients were calculated using Simple regression or for not normally distributed variables Spearman's rank correlation. The significance level was set at $p < 0.05$.

Results

Measurements of annexin V binding to endothelial cells

The frequency of HUVECs positive for annexin V staining was determined either as percentage of annexin V^+/ PI^- cells on a bivariate dot plot or percentage of annexin V^+ cells based on a histogram. Annexin V-binding to HUVECs in the presence of serum known to decreased binding and preincubated with IVIG was

determined. Preincubation with IVIG could restore binding of Annexin, indicating that antibodies present in IVIG could neutralize binding (Fig.4).

5   APC-BSA and aPC-KHL were both associated significantly in SLE-patients with a history of CVD with Annexin V binding to EC (r=0.45; p=0.02 and r=0.42 and p=0.03 respectively). aPC were determined as described above.

Effect on oxLDL uptake in macrophages by aPC

10   aPC of IgM and IgG subclass, extracted from IVIG as indicated were preincubated with oxLDL indicated (fig 3). We used total IgM as control for aPC IgM (macrophage+Dil-oxLDL+IgM) and effect on macrophage uptake. The total percentage of positive staining cells is 46.62%, indicating that IgM per se does not have the inhibitory effects that aPC has. IgM was bought from SIGMA, and is

15   purified human IgM is produced by precipitation and gel filtration techniques using normal human serum as the starting material. The immunoglobulin is determined to be at least 95% pure.

Effect of aPC on ICAM-induction in endothelial cells

20   PAF was incubated with EC at the indicated concentrations. As demonstrated in Fig 5 this lipid could induce a significant increase in ICAM-expression. aPC of IgM subclass, extracted from IVIG as indicated were preincubated with these lipids as indicated (Fig 5).

25

Correlations between aPC and other risk markers in ELSA study (226 individuals with hypertension as described previously.

aPC IgM was associated with two other protection factors, HSP 70 and HDL, as indicated in Table 4. There was also a weak albeit significant association with

30   TNF, a marker of inflammation and a proatherogenic cytokine.

TNF is an important pro-inflammatory cytokine and TNF levels negatively associated with aPC igM levels. The association is weak, but significant.

30

HSP 70 is a novel protection factor recently described by us and others. There is a clearly positive association. Also HSP60, which is a weaker protection factor, is associated.

HDL is a well known "good" cholesterol, with anti-inflammatory properties. It is associated significantly with aPC IgM.

			ANTPCIGG	ANTPCIGM
Spearman's rho	ANTPCIGG	Correlation Coefficient	1,000	,245
		Sig. (2-tailed)	,	,000
		N	220	220
	ANTPCIGM	Correlation Coefficient	,245	1,000
		Sig. (2-tailed)	,000	,
		N	220	220
	HDL	Correlation Coefficient	,008	,233
		Sig. (2-tailed)	,906	,001
		N	206	206
	TNFA	Correlation Coefficient	-,012	-,136
		Sig. (2-tailed)	,863	,044
		N	220	220
	HSP60	Correlation Coefficient	,138	,279
		Sig. (2-tailed)	,047	,000
		N	209	209
	HSP70	Correlation Coefficient	,157	,356
		Sig. (2-tailed)	,022	,000
		N	213	213

** Correlation is significant at the .01 level (2-tailed).
* Correlation is significant at the .05 level (2-tailed).